Preliminary evaluation of ethanol leaf extract of *Borreria verticillata* Linn (Rubiaceae) for analgesic and anti-inflammatory effects

Halima Sadiya Abdullahi-Gero¹*, Abubakar Ahmed², Abdulkadir Umar Zezi¹ and Isa Marte Hussaini³

¹Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.
²Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.
³Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Maiduguri, Borno State, Nigeria.

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*Borreria verticillata* L is used for the treatment of some painful and inflammatory conditions by traditional medical practitioners in Nigeria and other countries. The ethanol leaf extract of *B. verticillata* (EEBV) was investigated for possible analgesic and anti-inflammatory effects in mice and rats. The models used for the analgesic study were acetic acid induced abdominal writhes, hot plate tests in mice and formalin induced pain in rats. Carrageenan and formalin induced rat paw oedema were used to investigate anti-inflammatory effects. The oral (p.o) median lethal dose (LD₅₀) was greater than 5000 mg/kg body weight in mice and rats, while the intraperitoneal (i.p) LD₅₀ in mice was 3807.88 mg/kg and greater than 5000 mg/kg in rats. The results of the study showed that the extract to have significant (p<0.001) analgesic effect at dose range of 200 to 1000 mg/kg p.o/i.p in mice in the acetic acid induced writhes and hot plate tests. Significant (p<0.05) analgesic effect was observed at 500 and 1000 mg/kg p.o in both phases of formalin induced pain in rats. EEBV exhibited anti-inflammatory effects which were found to be significant (p<0.001, p<0.05) at doses of 200 to 1000 mg/kg p.o/i.p in the rats and in all models used.

**Key words:** *Borreria verticillata*, analgesic, anti-inflammatory.

INTRODUCTION

The use of medicinal plants for the relief and treatment of disease can be traced back to five millennia in various civilizations. Medicinal plants have played a vital role in world health (Calixto 2000; Calixto et al., 2000a). Despite the recent developments recorded in modern medicine, medicinal plants still make important contributions to health care (Calixto, 2000). In some African countries such as Ghana, Mali, Nigeria and Zambia, the first line of treatment for fevers resulting from malaria in about 60% of children, is the use of herbal medicines (WHO, 2003). Pain is an unpleasant sensory and emotional experience, associated with actual or potential tissue damage (IASP). Most people will experience pain at some time in their lives, because pain is a symptom that accompanies many...
ailments. Inflammation is the pathophysiological response of a living tissue to injury that leads to local accumulation of plasma fluids and blood cells (Mohanbabu et al., 2011). Currently, available drugs for the management of pain are opioids, non-opioid and non-steroidal anti-inflammatory drugs (NSAIDS). Prolong use of steroids (Corticosteroids) lead to several side effects including depression of the immune system. NSAIDs are used to manage acute mild to moderate pain and they have the potentials to cause severe adverse effects which include gastric mucosa ulceration and bleeding. Opioids are used for chronic agonizing and post operative pain and they have the potentials for addiction, dependence and tolerance (Almeida et al., 2001). The problems associated with the use of these drugs demands for research into plants with antioxidant properties that have folkloric use for the management of pain and inflammation with a view to finding new, safe and efficacious drug. Borreria verticillata has long history of use for several health conditions including fever and pain. Studies have investigated the antimicrobial activity of B. verticillata, but to the best of our knowledge none have been cited for the analgesic or anti-inflammatory activity of the plant.

This study therefore aims at examining the analgesic and anti-inflammatory effect of the ethanol leaf extract of B. verticillata (EEBV). The outcome of this study may explain the rationale behind the use of B. verticillata in the management of painful and inflammatory conditions by traditional medical practitioners as well as provide a baseline data on the safety or otherwise of the plant. B. verticillata is a shrub plant of the Rubiaceae family. It is known by the following common names; shrubby false button weed, shrubby false button wood (Burkill, 2000). In Nigeria, it is known as Karya garma (Hausa) Irawo ileye (Yoruba), Abia ikana (Ibibio). The flower is used as antipyretic and analgesic (Vieira et al., 1999; Moreira et al., 2010), the roots as anti diarrhoea and for treatment of dysentery and haemorrhoids (Lorenzi and Matos, 2002). In West India, the decoction of the plant is used for diabetes and dysmenorrhoea. It is used in combination with Cuscuta and Zebrina schnizlein for the treatment of amenorrhoea (Ayenu, 1978; Conserva and Ferreira, 2012). B. verticillata is used to treat bacteria skin infections and leprosy in Senegal (Sofowora, 2008). The juice of fresh aerial part is used in Nigeria for the treatment of skin eczema (Benjamin, 1979; Ajibesin et al., 2008). Essential oils isolated from B. verticillata have been shown to inhibit the growth of Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans (Burkill, 2000; Ushie and Adamu, 2010).

MATERIALS AND METHODS

Drugs and chemicals

The following drugs and chemicals of analytical grade were used:

- Ethanol (Sigma Aldrich USA), N-hexane (Sigma Aldrich USA), Piroxicam (Hovid, Malaysia), Morphine (Martindale, Essex), Carrageenan (Sigma Aldrich U.S.A), Formaldehyde (BDH, Chemical Laboratory, England, UK).

Equipment

- Hot plate (22/SS – 615, Gallenkamp, England), weighing balance (Metttler, P152), stop watch, needles and syringes, spatula, animal cages, pestle and mortar, test tubes, beakers, digital vernier calliper (Precision measuring, SR44), gloves and cotton wool.

Plant

The whole plant of B. verticillata was collected from Basawa in Zaria, Kaduna State, Nigeria. The plant was identified and authenticated by Mallam Umar Gall Department of Biological Sciences, ABU, Zaria, Nigeria. A voucher specimen number (672) was deposited at the herbarium for future reference. The leaves were then picked and dried under shade until constant weight was obtained. The dried leaves were then crushed into coarse powder using a pestle and mortar.

Extraction

The powdered leaves (200 g) were extracted with 1.2 L of N-hexane. The marc was extracted with 1.2 L of aqueous ethanol (70% ethanol and 30% water, v/v) for 24 h at room temperature using a percolator. The solvent was removed over a water bath at temperature 45°C. The extract was stored in a closed container and referred to as EEBV.

Phytochemical screening

Preliminary phytochemical screenings were carried out on EEBV in order to confirm the presence of phyto-constituents following the methods described by Sofowora (2008) and Evans (2009).

Acute toxicity studies in rats and mice (LD50)

The determinations of LD50 were conducted following the method of Lorke (1983), in both rats and mice and by oral (p.o) and intraperitoneal (i.p) routes. The method was divided into two phases. In the first phase, 3 groups of three animals each received the plant extract of B. verticillata (EEBV) in doses of 10, 100 and 1000 mg/kg body weight and was observed for sign of toxicity and death for 24 h. In the second phase, 3 groups of one animal each received a more specific dose of the extract (1600, 2900 and 5000 mg/kg). The LD50 was determined by calculating the geometric mean of the lowest lethal dose and the highest non lethal dose (1/1 and 0/1), that is, LD50 = √ (Highest non lethal dose) × (Lowest lethal dose).

Analgesic activity

Hot plate test

The analgesic activity of the extract was measured by hot plate method as described by Eddy and Lambach (1953) and Vogel (2002). Thirty pre-screened (reaction time: 3 to 4 s) Swiss albino mice were randomly divided into 5 groups each composing of 6 mice. Group 1 received normal saline 10 ml/kg body weight, groups
The reaction time was taken as from the time the animal was placed on the hot plate to the time it licked its paw or jumped out of the hot plate. The reaction time for the mouse to lick its paw or jumped was taken as pain reaction time. The reaction time was recorded at 0, 30, 60, 120, and 180 min following treatments with extract, control or standard drug. A cut off time of time of 20 s was set to prevent injury to the animal. The percent analgesia was calculated, thus:

\[
\text{Analgesia} \% = \frac{\text{Latency test} - \text{Latency control}}{\text{Latency control}} \times 100
\]

The same procedure was repeated with another set of animals, but animal received treatments orally.

**Formalin induced pain in rats**

Thirty six albino rats were divided into 6 groups of 6 rats each and were administered either normal saline of 10 ml/kg oral, EEBV (250, 500 and 1000 mg/kg), morphine 5 mg/kg or piroxicam 20 mg/kg oral. One hour after treatments, 0.05 ml of freshly prepared formalin of 2.5% was injected subcutaneously into the sub plantar region of the left hind paw of each rat. The rats were placed individually in an observation chamber and monitored for 1 h. The severity of pain response was recorded for each rat based on the following Likert scale. Rat walked or stood firmly on injected paw (0); the injected paw was partially elevated (1); the injected paw was clearly lifted off the floor (2); and the injected paw was licked, chewed or shook (3). The anti-nociceptive effect was determined in 2 phases. Phase 1 was recorded during the first 5 min and phase 2 during the last 45 min with 10 min lag period in between the two phases (Dubuisson and Dennis, 1977; Tjolsen et al., 1992).

**Carrageenan induced paw oedema**

Thirty albino rats were randomly divided into 5 groups of 6 rats each. Acute inflammation was induced by injecting 0.1 ml of 1% saline solution of carrageenan into the sub plantar surface of each rat hind paw (Winter et al., 1962). Normal saline (10 ml/kg), EEBV (250, 500 and 1000 mg/kg) and piroxicam 20 mg/kg were administered to the animals 30 min before carrageenan injection (for intraperitoneally) and 1 h before carrageenan injection (for oral treatment). The paw diameter was measured at 0, 1, 2, 3, 4 and 5 h following carrageenan injection. The differences between reading at 0 h and at different time intervals were taken as the diameter of oedema.

**Formalin induced inflammation**

Thirty albino rats were divided randomly into 5 groups of 6 rats. The first group served as negative control and received normal saline of 10 ml/kg, while groups 2, 3 and 4 received different doses of the extract (250, 500, 1000 mg/kg) body weight orally. The fifth group received piroxicam (20 mg/kg) body weight (intraperitoneally). One hour later, all groups were administered 50 µl of 2.5% solution of formalin subcutaneously into the sub plantar region of the left hind paw of each rat. An increase in hind paw diameter induced by formalin was used to measure acute inflammation (Winter et al., 1963). The paw diameter was measured with the aid of a digital vernier caliper at 0, 1, 2, 3, 4 and 24 h after the injection of formalin. The differences between the readings at time 0 h and at different time intervals were taken as the diameter of oedema (Hess and Milong, 1972).

**Statistical analysis**

All data obtained were expressed a mean ± standard error of mean. Data were analysed by one way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) version 19 followed by Turkey’s Post Hoc test for hot plate test, acetic acid induced writhes test, carrageenan and formalin induced paw oedema and independent samples Kruskal-Walis and Mann Whitney’s test for formalin induced pain. Values of p<0.05 were considered significant. Data were presented in form of figures, graph and tables.

**RESULTS**

**Preliminary phytochemical screening**

The preliminary phytochemical screening revealed the presence of alkaloids, saponins, phenol, tannins, glycosides, sterols/terpenoids, carbohydrates and flavonoids.

**Median lethal dose (LD50)**

The intraperitoneal and oral median lethal doses (LD50) of ethanol leaf extract of *B. verticillata* in rats were found to be greater than 5000 mg/kg body weight. The oral LD50 in mice was also above 5000 mg/kg, whereas the intraperitoneal LD50 in mice was 3807.88 mg/kg body weight.
Analgesic studies

The effect of ethanol leaf extract of B. verticillata on acetic acid-induced writhes in mice

The extract significantly (p<0.001) inhibited the number of acetic acid-induced writhes in mice in a dose-dependent manner by both intraperitoneal and oral route of administration. The highest percentage of inhibition (90.21%) of writhes response was observed with ethanol leaf extract of B verticillata (EEBV) 1000 mg/kg (oral). Piroxicam, the standard drug, showed a maximum writhes inhibition of 76.00%. The lower doses of the extracts 200 (intraperitoneally) and 250 mg/kg (orally) showed higher percentage writhes inhibition of 78.85 (intraperitoneally) and 79.12 (orally) compared with piroxicam of 74.99 (intraperitoneally) and 76.0 (orally), respectively (Figure 1).

The effect of EEBV on thermally-induced pain stimulus in mice

EEBV significantly (p<0.001) elevated the reaction time to thermal stimulus in mice in a dose dependent manner. The highest protection was observed with morphine at 180 min in both oral (Figure 2) and intraperitoneal (Figure 3) routes were 126.94 and 128.57%, respectively. The reaction time at dose of 800 and 1000 mg/kg were found to be the highest at 180 min (Figures 2 and 3).

The effect of EEBV on formalin induced pain

The extract at doses of 500 and 1000 mg/kg significantly (p<0.05) reduced the severity pain in both phases I and II, while at 250 mg/kg the reduction in pain severity was not significant (Figure 4). Morphine 5 mg/kg, the standard drug used in the study inhibited both phases of nociception, while piroxicam inhibited only the second phase significantly (Figure 4).

The effect of EEBV on carrageenan-induced paw oedema in rats

The injection of 1% saline carrageenan solution into the
sub plantar region of the hind paw of the normal saline (negative control) treated group produced local oedema reaching its maximum at the fourth hour. EEBV, at all doses (250, 500 and 1000 mg/kg oral) significantly (p<0.001) inhibited the paw oedema. However, maximum oedema inhibition was observed at dose of 500 mg/kg (57.42%) in the fifth hour (Figure 5). The percentage anti-inflammatory effect of piroxicam at the time of maximum oedema inhibition was 64.86% (Figure 5).

The effect of intraperitoneal administration of EEBV on carrageenan-induced paw oedema in rats

The injection of 1% saline carrageenan solution into the sub plantar region of the hind paw of the normal saline (negative control) treated group produced local oedema reaching its maximum at the third hour. EEBV, at all doses (200, 400 and 800 mg/kg) significantly (p<0.001) inhibited the paw oedema. However, maximum oedema inhibition was observed at dose of 800 mg/kg (55.77%) in the fourth hour (Figure 6). The percentage anti-inflammatory effect of piroxicam at the time of maximum oedema inhibition was 59.36% (Figure 6).

The effect of EEBV on formalin-induced paw oedema in rats

The injection of formalin into the sub planter region of the rats’ hind paw produced a localized oedema, evident at the first hour and reached peak at the 24th hours in the control group. EEBV significantly (p<0.001) inhibited the paw oedema at doses (250, 500 and 1000 mg/kg oral). The maximum inhibition (63.86%) was observed with EEBV (1000 mg/kg) at the 24th hour when compared with the control group. Piroxicam, a standard anti-inflammatory drug produced 66.92% inhibitions at 24th hour (Figures 4 to 7). The inhibition of paw oedema produced by EEBV was comparable to that of piroxicam, the standard anti-inflammatory drug used in the study (Figure 7).
DISCUSSION

The preliminary phytochemical screening of *B. verticillata* showed the presence of alkaloids, saponins, steroids, glycosides, flavonoids, tannins and phenols. This finding is consistent with those of Ushie and Adamu (2010) and Conserva and Ferreira (2012). These compounds have well known pharmacological activities including analgesic, anti-inflammatory and antioxidant effects (Perez, 2001; Park et al., 2001). A number of flavonoids as well as tannins isolated from plants have been discovered to have significant analgesic and/or anti-inflammatory effects (Duke, 1992; Ahmadiani et al., 1998, 2000). The inhibitory effect of flavonoids on eicosanoids synthesis has been documented (Damas et al., 1985; Raj et al., 2001; Danjuma et al., 2011). The ability of certain flavonoids to inhibit a wide array of enzymes such as protein kinase C, protein tyrosine kinases, phospholipase A₂, phosphodiesterases and others have been reported (Middleton, 1998). Alkaloids and saponins possess analgesic and antispasmodic effects, while the healing of wounds and inflamed mucus membranes are hastened by tannins (Okwu and Okwu, 2005). Thus, the presence of these constituents may be contributory to the analgesic and anti-inflammatory effects of the plant.

The oral median lethal dose (LD₅₀) of the extract in both mice and rats were greater than 5000 mg/kg, the intraperitoneal LD₅₀ was greater than 5000 and 3807.88 mg/kg in rats and mice, respectively. This suggests that the extract is relatively non toxic orally and may be slightly toxic when administered intraperitoneally. This analysis is based on the toxicity classification proposed by Loomis and Hayes (1996) which states that substances with an LD₅₀ values between 500 and 5000 mg/kg
Figure 4. Effects of oral administration of EEBV on formalin-induced pain in rats. *p<0.05 compared to normal saline, **p<0.05 (n=6) compared to morphine, not significant compared to piroxicam, Kruskal wallis followed by Mann Whitney test. EEBV = Ethanol leaf extract of *B. verticillata*, N/S = Normal Saline and PRM = Piroxicam.

Figure 5. Effect of oral administration of EEBV on carrageenan induced paw oedema in rats. *p<0.001, **p<0.05 compared to normal saline, ***p< 0.05 (n=6) significant compared to piroxicam, Turkey’s post hoc test. EEBV = Ethanol leaf extract of *B. verticillata*, N/S = Normal Saline and PRM = Piroxicam.
and 5000 mg to 15,000 mg/kg body weight are regarded as slightly toxic and practically non toxic, respectively.

Four different animal models were employed to investigate the potential analgesic and anti-inflammatory activities of the plant extract. The models selected enabled the investigation of both central and peripherally-mediated effects.

The acetic acid-induced writhes response is a procedure to evaluate peripheral acting analgesics (Gene et al., 1998). The ethanol leaf extract of *B. verticillata* (EEBV), significantly (*p*<0.001) and dose-dependently inhibited acetic acid-induced writhes response in mice at doses investigated both by oral and intraperitoneal routes of administration. The acetic acid-induced writhes test is a sensitive test for the screening of peripheral analgesic activity of compounds (Gene et al., 1998). Increased level of prostanoids such as prostaglandins E₂ (PGE₂) and F₂ (PGF₂) in the peritoneal fluid has been implicated in the acetic acid writhes test (Deraedt et al., 1980). Some researchers have also postulated the involvement of lipooxygenase (LOX) and cyclo-oxygenase (COX) (Levine et al., 1984; Dhara et al., 2000). Local peritoneal nociceptive receptors are presumed to be partly involved in the abdominal constriction response (Bentley et al., 1983). The high sensitivity of the acetic acid-induced writhes test allows for detection of nociceptive activity of compounds at dose levels that might seem inactive in other methods like the tail flick test (Collier et al., 1968; Bentley et al., 1981).

The hot plate test described by Eddy and Lambach (1953) is suitable for the evaluation of centrally acting analgesics. Thermally induced nociception suggests narcotic involvement (Besra et al., 1996). The pain threshold in mice is generally elevated by centrally acting analgesics. The nociceptors appear to be sensitized by sensory nerves and endogenous peptides such as prostaglandins may have minimal involvement in this model (Mohan et al., 2009).

Another reliable method of evaluating analgesic activity of compounds is the formalin test. It is better correlated with clinical pain (Tjolsen et al., 1992; Ghannadi et al., 2005). The formalin induced pain elucidates both central

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**Figure 6.** Effect of intraperitoneal administration of EEBV on carrageenan induced paw oedema in rats. *p*<0.001, *p*<0.05 compared to normal saline, *p*<0.05 (n=6) significant compared to piroxicam, Tukey’s post hoc test. EEBV = Ethanol leaf extract of *B. verticillata* N/S = Normal Saline and PRM = Piroxicam.
and peripheral mediated mechanism. The formalin test showed a unique biphasic responses termed early and late phases. The early phase response represents a direct chemical stimulation of pain due to the irritant effect of formalin on sensory C fibres (Hunskaar et al., 1985; Tjolsen et al., 1992) whereas the late phase response is due likely to the development of an inflammatory response and the release of allergic mediators such as serotonin, histamine and bradykinin (Hunskaar and Hole, 1987). Drugs that act centrally inhibit both phases, while those that act peripherally inhibit only the late phase (Chen et al., 1995). The significant (p<0.05) suppression of both phases of pain observed with EEBV (500 and 1000 mg/kg) indicates that the extract has both central and peripheral analgesic effect. This inference is further strengthened by the significant activity observed with both acetic acid writhes and the hot plate tests.

Oedema induced by phlogistic agents is a widely accepted model for the evaluation of the anti-inflammatory effect of drugs (Winter et al., 1962; El-Shenawy et al., 2002). To evaluate the anti-inflammatory activity, EEBV was assessed using two popular screening models widely used for NSAIDs, namely, carrageenan and formalin-induced rat paw oedema.

Carrageenan-induced hind paw oedema is the standard experimental model for acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effect (Chakraborty et al., 2004). The model is also known to show a high level of reproducibility (Winter et al., 1962). The probable mechanism of action of carrageenan is biphasic (Vinegar et al., 1969). The first phase is due to the release of histamine and serotonin (0 to 2 h), kinin like substances maintains the plateau phase (3 h) and the second phase is due to the release of prostaglandins (Perianayagam et al., 2006; Fotio et al., 2009). In the carrageenan-induced rat paw oedema test, EEBV at 500 mg/kg body weight showed slightly higher effect than 1000 mg/kg orally. This may be as a result of possible biological variation within test groups and possible interactions between constituents of the extract.

The formalin-induced rat paw oedema also showed a biphasic response and may originate mainly from neurogenic inflammation (up to 24 h) followed by the participation of kinins and leukocytes with their pro-inflammatory factors including prostaglandins (Wheeler- Aceto and Cowan, 1991; Popov et al., 2005). Inflammation induced

*Figure 7. Effect oral administration of EEBV on formalin induced paw oedema in rats. *p<0.001, (n=6) compared to normal saline, not significant compared to piroxicam, Turkey’s post hoc test. EEBV = Ethanol leaf extract of B. verticillata, N/S = Normal Saline and PRM = Piroxicam.
by formalin results in cell damage leading to the production and release of endogenous mediators like histamine and bradykinin. The response observed with formalin test correlates with that of carrageenan-induced rat paw oedema, thus re-affirming the anti-inflammatory effect of the plant extract.

The presence of both anti-inflammatory and analgesic effects seen with the extract (EEBV) is well defined for a variety of NSAIDs, especially the salicylates and their derivatives. It is therefore fascinating that the extract behaved like an NSAID in the study and this correlates with the traditional application of the plant in the management of painful and inflammatory conditions. The differences in activity of the extract at, 250 mg/kg body weight in the formalin-induced pain compared to those observed with the hot plate and the acetic acid induced writhes test could also be attributed to possible biological variation within the test groups. The visible lack of significant difference in the effects of different doses in some of the tests for example hot plate (orally and intraperitoneally) could be attributed to a ceiling effect at 250 mg/kg after which increase in dose may not result in any significant increase in observed effects (Vongtau et al., 2004).

Substances are administered to laboratory animals through several routes including oral and intraperitoneal routes. The oral route is economical, convenient and relatively safe; moreover, when substances are investigated for safety, the oral route mimics the common mode of use in humans. Substances administered by oral route may be poorly absorbed or inactivated by gastric secretions in the stomach and possibly first pass effect in the liver. Intraperitoneal route of administration provides faster absorption through the large vascular beds of the intestines. The intraperitoneal route is also accepted for absorption through the large vascular beds of the peritoneum. The intraperitoneal route is also accepted for intraperitoneal administration and no difference in responses were observed, suggesting the possible similarity in oral and intraperitoneal pharmacokinetic profile of EEBV.

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Conflict of Interest

Authors declare no conflict of interest.

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